Composition Studies on Tobacco. IV. Ergosterol, γ-Sitosterol and a Partially Characterized Steroidal Glycoside from Flue-cured Leaves

with that of the epimer of γ-sitosterol, β-sitosterol. Unfortunately, an authentic sample of the γ-epimer was not available for comparative purposes. However, the above data would seem sufficient to identify the compound as γ-sitosterol.

Group II. Solid IIA was the initial precipitate obtained on the addition of water to an ethanolic solution of the acetates of Group II. IIA was recrystallized once from ethanol and then gave m.p. 163°-165° C, $[\alpha]^{20}D$ —119. The ultraviolet spectrum of IIA showed the absorption maxima at 271, 282 and 293 mμ characteristic of ergosterol. Calculated from the molecular weight of ergosteryl acetate, the molecular extinction coefficient of IIA in methanol was 11,800 at 282 mμ, which is equivalent to 10,680 for the free sterol (lit., ergosterol: ε = 10,600 at 282 mμ (Hogness *et al*, 1937)). No depression of melting point was observed on admixture of IIA with authentic ergosteryl acetate. IIA was saponified and a sterol, m.p. 153°-155° C, $[\alpha]^{20}D$ —134, was obtained which showed an infrared spectrum identical with that of authentic ergosterol.

Solid IIB, which was too small in quantity for further work, was obtained on evaporation of the filtrate from the precipitation of IIA. IIB melted near 145° C and showed infrared spectral characteristics similar to IA2.

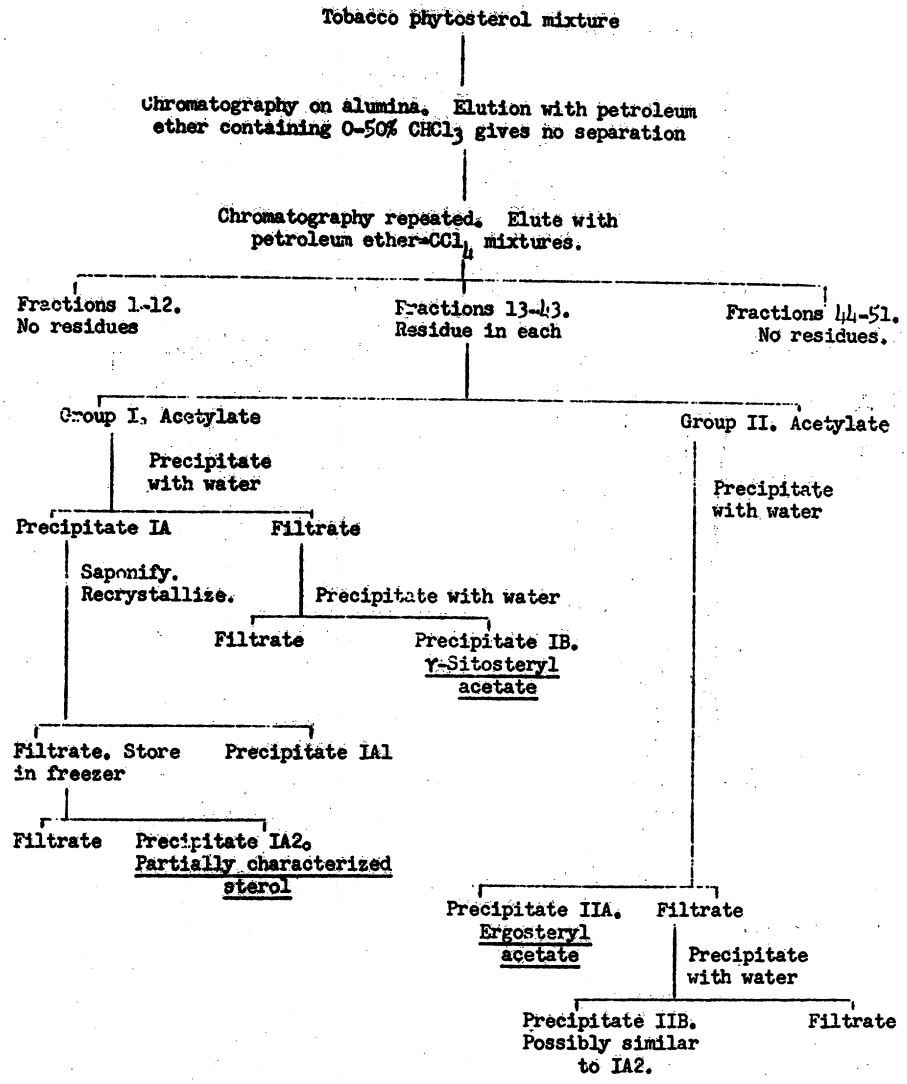

Figure 1. Fractionation of mixed phytosterols obtained from flue-cured tobacco leaves.

Discussion

Although the identity of ergosterol is conclusively established by the above data, a comparison of the melting points of the tobacco sterol and its acetate with values from the literature for ergosterol and ergosterol acetate has shown discrepancies. This is understandable since ergosterol is quite difficult to obtain in high purity. As stated in previous papers in this series, the specific optical rotations and spectral characteristics of isolated phytosterols are frequently more reliable criteria for identification than are melting points. The absence of conjugated sterols in flue-cured tobacco was reported in Part III. The evidence for this claim was based on a negative Rosenheim test. Further work has shown that the von Christiani and Anger test (1939) is more than 20 times as sensitive as the original Rosenheim method and gives a positive reaction with the mixed tobacco phytosterols. Additional evidence that ergosterol is a minor component of the steroidal complement of flue-cured tobacco is shown by the failure to obtain an ultraviolet absorption spectrum with the mixed tobacco phytosterols at high concentrations (up to 0.8 g mixed sterols per l.)

The Partially Characterized Sterol. In the above work (Precipitate IA2) and in Part II (Grossman and Stedman, 1958), brief reference was made to the isolation of a tobacco sterol which is similar to but not identical with stigmasterol. This sterol is apparently a compound not previously reported in plants. Unfortunately, complete characterization has not been achieved because of limited available material. The following presentation summarizes the work performed thus far and is given to assist workers who may isolate adequate quantities of the compound in the future.

Isolation

The sterol has been isolated in two instances. Small amounts were obtained above as Precipitate IA2; since the original phytosterol mixture yielding IA2 was isolated from tobacco extracts successively hydrolyzed with acid and alkali, the sterol might have occurred as a glycoside or ester in the leaves. Larger amounts of the sterol in glucosidated form have been obtained from a fractionating scheme identical, in part, to that described in Part I, and this material has been employed in all work on characterization reported herein. The glucoside was obtained from an ethanolic extract of unaged, flue-cured tobacco which had been previously extracted with Skellysolve B. Water saturated with sodium chloride was added to the ethanolic solution which was then extracted with diethyl ether. The ether solution was evaporated to 25 per cent of the original volume and washed with acid and water. On shaking the ether solution with water, a flocculent precipitate characteristic of steroidal glycosides formed at the interface and was filtered off. The precipitate was successively washed with small amounts of chloroform, dioxane, diethyl ether, methanol and ethanol. The residue remaining after

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Introduction

Part III of the current series described a quantitative study of total phytisterols in flue-cured tobacco (Stedman et al 1958). These mixed sterols have now been partially separated into individual components, two of which, γ-sitosterol and ergosterol, are described in this report. Although the occurrence of ergosterol in tobacco has not been previously demonstrated, the presence of γ-sitosterol in processed chewing tobacco has been shown by Khanolkar et al (1955).

The present report also includes the partial characterization of a new plant steroidal glycoside isolated from flue-cured tobacco.

Experimental, Results and Discussion

Ergosterol and γ-Sitosterol

Isolation and Identification. The essential steps in the fractionating scheme are presented in **Figure 1**; details of the method are as follows: Initially, 2.7 g of mixed phytosterols (obtained from unaged, flue-cured tobacco as described in Part III) were dissolved in chloroform and added to a 22 x 460 mm chromatographic column of Merck[2] acid-washed alumina. No separation of individual components was achieved on collection of 120 fractions eluted with 1l. solvents in a flowing technique (petroleum ether containing 0-50 per cent chloroform). The residues (total, 2.7 g) were combined and 255 mg were again added to an alumina column. Petroleum ether containing 1-6 per cent carbon tetrachloride failed to elute any solids (fractions 1-12, 50 ml each). On gradually increasing the concentration of carbon tetrachloride from 6-25 per cent by 1 per cent increments (100 ml of each increment), residues were obtained in fractions 13-43 (containing 50 ml each) but not in fractions 44-51.

The melting points[3] of the residues were determined. On the basis of similarities in melting points, 19 of the fractions were pooled into two groups: the residues of Group I (65 mg total) melted at 139°-141° C; those of Group II (40 mg total) at 147°-151° C. On recrystallizing once from ethanol, Group I had m.p. 142°-144° C, $[\alpha]^{20}_D$ —35.2[4] and Group II gave m.p. 148°-149° C, $[\alpha]^{20}_D$—134. The high negative rotation of Group II indicated the presence of a sterol possessing conjugated double bonds in ring B.

Each group was then recrystallized three more times from ethanol, the sterols were acetylated and the acetates subjected to fractional precipitation by the addition of water to ethanolic solutions of the derivatives. The following results were obtained:

Group I. Solid IA, m.p. 134°-134.5° C, $[\alpha]^{20}_D$—41.2, was obtained on initial addition of water. IA was saponified, and the free sterol (IA1) recrystallized from ethanol. On storage (at —20° C overnight) of the filtrate resulting from this recrystallization, precipitate IA2 resulted. IA1 showed m.p., 149°-151° C, $[\alpha]^{20}_D$ —35.8 and was too small in quantity for further work. IA2 gave m.p. 143°-144° C, $[\alpha]^{20}_D$—39.0. The infrared spectrum was similar to that of stigmasterol and was identical with that of the partially characterized sterol discussed later.

Solid IB was obtained on further addition of water to the filtrate resulting from the separation of IA. IB gave m.p. 139°-140° C, $[\alpha]^{20}_D$—45 on recrystallization from ethanol (lit., γ-sitosteryl acetate: m.p. 140° C (Khanolkar *et al*, 1955), $[\alpha]^{20}_D$—47.7 (Heilbron *et al*, 1943)). On saponification of the acetate and recrystallization from ethanol, a sterol, m.p. 149°-150° C, $[\alpha]^{20}_D$—44.6 was obtained (lit., γ-sitosterol: m.p. 148° C, $[\alpha]^{20}_D$—43.8 (Khanolkar, 1955)). Benzoylation of the sterol gave a deriative, m.p. 150°-151° C, $[\alpha]^{20}_D$ —19.1 (lit., γ-sitosteryl benzoate: m.p. 152° C, $[\alpha]_D$—19.6 (Heilbron *et al*, 1943)). The infrared spectrum of the above free sterol was identical

[1] *Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.*
[2] *Use of a specific commercial product does not constitute endorsement by the United States Department of Agriculture.*
[3] *The Fisher-Johns melting point apparatus was used for all determinations reported herein.*
[4] *All rotations were determined in chloroform.*

tobacco sterol. Likewise the possibility of a mixture of known compounds appears unlikely since the melting points of possible combinations of known Δ^5 compounds derived from campestanol should be lower than that of the tobacco sterol. According to our present knowledge, unsaturation occurs only at the Δ^{22} and $\Delta^{24(28)}$ positions in the side chains of Δ^5 sterols derived from campestanol. The infrared spectrum of the tobacco sterol would indicate that unsaturation at Δ^{22} probably exists.

Thus the sterolin isolated from tobacco appears to be a glucoside of a Δ^5 sterol having a typical phytosterol side chain with a 24a-methyl configuration. An examination of the above saponification equivalent obtained on splitting of the acetylated sterolin shows that, for the above conditions to be met, the sterolin must be a monoglucoside.

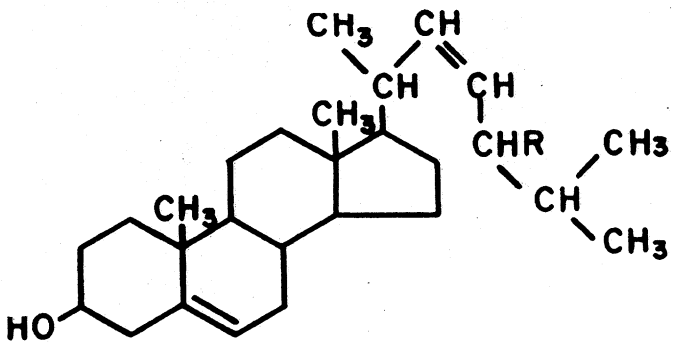
A comparison of literature values for known phytosterols of the above types with the tobacco sterol has shown that one known compound, chalinasterol, compares favorably in physical properties with the tobacco sterol in all but one instance (see table 1 and below). Chalinasterol has been reported as occurring in sponges and sea anemones by Bergmann and co-workers (Bergmann *et al*, 1945, 1951).

The structure originally proposed by Bergmann and co-workers for chalinasterol is shown below. If the tobacco sterol possesses this structure the spectral indications of Δ^{22} unsaturation would be expected.

Recently, however, Bergmann and Dusza (1957) have revised their proposed structure of chalinasterol. They now believe that chalinasterol is 24-methylene cholesterol, differing from the originally proposed structure in that the unsaturation has been changed from Δ^{22} to $\Delta^{24(28)}$. These workers have also indicated that their original "chalinasterol" was probably a mixture which contained significant amounts of the epimer of brassicasterol[6] (III), the latter being responsible, presumably, for the optical rotations used in postulating the original structure. The physical constants of chalinasterol reported in 1957 are identical with those listed for the original material isolated some years ago. If the original material of these workers was a mixture and their current material is a pure compound, it is difficult to understand how the melting points of the two substances and derivatives thereof can be the same.

The tobacco sterol is not identical with 24-methylene cholesterol since the infrared spectra of the two are significantly different. Complete characterization of the tobacco sterol must await elucidation of the side chain. Unfortunately, the amount of material available has not permitted completion of this work. Oxidation with permanganate by a special method has not been successful; destruction of the sterol has occurred with both the unidentified sterol and stigmasterol run as a model compound. Insufficient material has prevented the use of ozonolysis in which losses of material by side reactions are usually great.

[6] *This epimer might be expected to have an infrared spectrum similar to stigmasterol. The epimer has not been isolated and characterized as yet.*



I = R = 24a-METHYL = CHALINASTEROL
(originally proposed structure)

II = R = 24b-ETHYL = STIGMASTEROL

III = R = 24b-METHYL = BRASSICASTEROL

Figure 3. Structures of chalinasterol, stigmasterol and brassicasterol.

Summary

A partial separation of mixed tobacco phytosterols has been achieved by column chromatography on acid-washed alumina followed by fractional precipitation; ergosterol and γ-sitosterol were isolated and identified. Ergosterol is apparently a minor component of the phytosterol mixture. A previous claim that conjugated sterols are absent in such tobacco was due to the comparative insensitivity of the color test employed.

The isolation of a previously unreported steroidal glucoside from flue-cured tobacco is described. The sterol possesses Δ^5 unsaturation, a 24a-methyl configuration and, probably, unsaturation in the side chain. The physical constants for the sterol are similar to chalinasterol, but the infrared spectra of the two compounds are different. Failure to characterize completely the structure of the sterol has been due to the limited amounts of available material.

Acknowledgments

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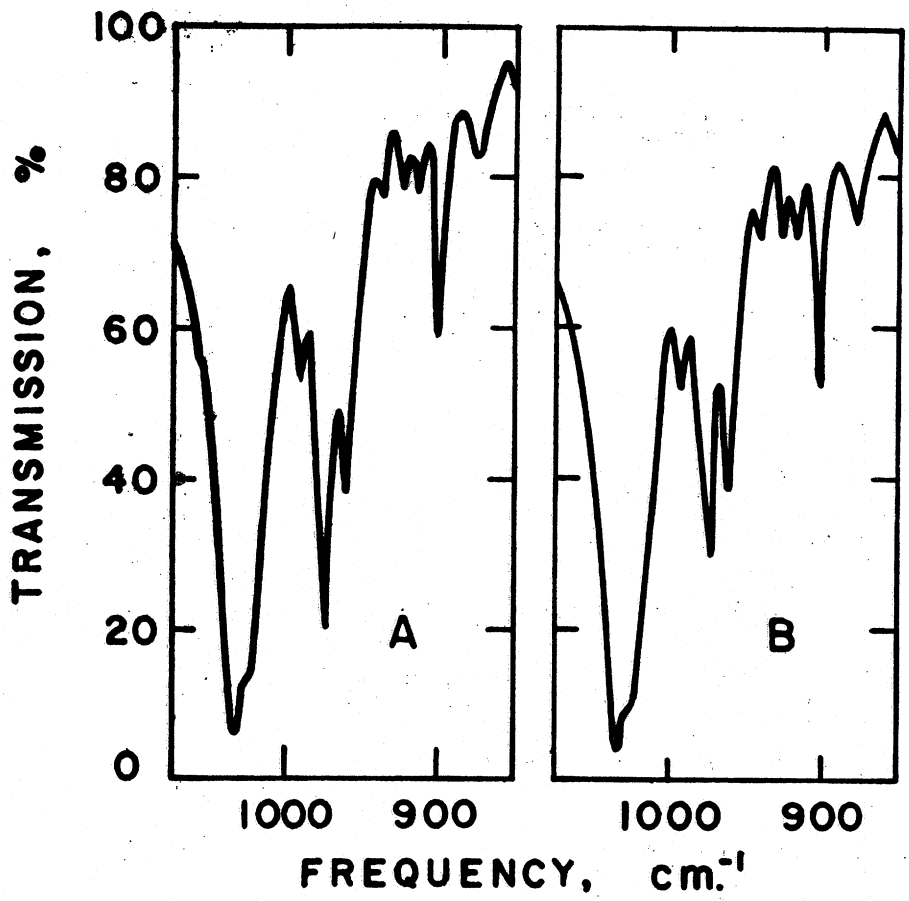

Figure 2. Infrared spectra of stigmasteryl acetate (A) and acetate of partially characterized tobacco sterol (B).

these washings contained the glycoside in question. The infrared spectrum had absorption bands at 960 and 972 cm^{-1} characteristic of stigmasterol but showed a higher degree of hydroxylation than is characteristic of stigmasterol.

Physical Characteristics. The residue was recrystallized from glacial acetic acid several times and then acetylated. The melting point and optical rotation of the acetate after recrystallization from methanol are given in **Table 1.** Saponification of the acetylated derivative gave an equivalent of 704. The liberated compound had the melting point and optical rotation listed in **Table 1** after recrystallization from glacial acetic acid. Analysis of this compound by x-ray diffraction indicated the absence of polymeric substance and of straight chains of more than approximately four carbon atoms. All of this evidence indicated the strong possibility that the substance was (or contained predominately) a glycoside of a sterol similar to stigmasterol.

Table 1. Melting points (°C) (MP) and specific optical rotations (SOR) of the partially characterized sterol from tobacco and of chalinasterol

Compound	Present Study MP	Present Study SOR	Bergmann et al* MP	Bergmann et al* SOR
Glycoside	289 -291	—	—	—
Glycoside tetraacetate	160 -161.5	—21.1		
Sterol	145 -146	—41.8	147-147.5; 144	—42
Acetylated sterol	137.5-138	—46.8	136	—46
Benzoylated sterol	143 -145	—19.2	145; 151	—19.4
Campestanol	144.5	+27.1	145	+26

* *From Bergmann et al, 1945, 1951. All determinations of specific rotation were made in chloroform.*

terol.

The substance was hydrolyzed with weak acid and a white solid giving a positive Liebermann-Burchard test was obtained on addition of water to the hydrolysis mixture. The solid was acetylated, and an acetate with the melting point and optical rotation given in **Table 1** was obtained. The infrared spectrum of this compound was almost identical with that of stigmasterol acetate. Differences in intensity were noted in the 850-1000 cm^{-1} region, including a weaker absorption for the tobacco sterol in the 972 cm^{-1} band indicative of *trans* Δ^{22} unsaturation. **Figure 2** illustrates these differences.

The acetate was saponified and, from the saponification mixture, a sterol was recovered which had the melting point and optical rotation listed in **Table 1** after several recrystallizations from ethanol. On benzoylation of the compound, a derivative having the physical properties presented in **Table 1** was obtained. Catalytic hydrogenation of the compound and recrystallization of the hydrogenated product from chloroform gave pure campestanol, m.p. 144.5° and $[\alpha]^{20}D+27.1$.

The filtrate obtained from precipitation of the sterol in the above hydrolysis mixture was chromatographed on paper, and the presence of a single sugar giving an R_F indentical with glucose was observed. The identity of the sugar was confirmed by conversion to glucosazone in the usual way.

Discussion of Structure

From the above data, some structural features of the sterol can be established. The optical rotation of the free sterol is witin the range of values for Δ^5 compounds. In addition, differences in molecular rotations of the sterol[5] and its derivatives based on the above data fall within the range accepted for Δ^5 sterols (Fieser and Fieser, 1949).

The compound belongs to the campestanol series, having a 24a-methyl configuration. Isolation of pure campestanol from the hydrogenated mixture shows that the material is a pure compound or a mixture of Δ^5 compounds differing only in the degree or position of unsaturation in the side chains. The possibility of the side chain being saturated is excluded since this compound would be campesterol, the physical constants of which do not match the

[5] *The molecular weight used in these calculations was derived from the saponification equivalent (see Discussion).*

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